# Establishment of a DNA vector system for doxycycline-inducible and β-cell-specific transgene expression (1)

Jiun-Shuan Chao (2) (4) Chiun-Jye Yuan (3) and Yu-Shine Jea (2)

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## Abstract

A single vector containing a complete tetracycline-on (Tet-on) inducible system was proposed and constructed with both regulatory and responsive elements. The developed Tet-on inducible vectors contain a Tet-sensitive transactivator gene (rtTA) under the control of the rat insulin II gene promoter (RIP) or porcine insulin gene promoter (PIP), a transgene cloning site, which is under the control of Tet operator sequence (tetO) flanked with a minimal CMV promoter (TetCMVmin). A neomycin resistant gene expression cassette was also included for screening stable clones in the future. With the developed Tet-on inducible vector, the green fluorescent protein (GFP) as a reporter gene could be effectively induced in HIT-T15 cells, a hamster pancreatic β-cell line, by the treatment of doxycycline (Dox), and is also responsive to glucose in the regulation of transgene expression, demonstrating the versatility of this single vector-based inducible system in β-cell specific expression of transgene, whose transactivator is controlled by RIP or PIP. In contrast, it could not lead to the expression of GFP in DK cell (duck kidney cell line) in the presence of Dox. In conclusion, the developed Tet-on inducible vector exhibits a high potential in controlling transient gene expression and the generation of transgenic animal models.

Key words: Tetracycline inducible system, β cells, Gene regulation.

## Introduction

Transient expression is essential for studying the function of specific transgenes in the specific cells or animal models. However, the uncontrolled expression of transgenes may exhibit undesirable side effects. This is particularly true for the gene products with cytotoxicity or growth inhibitory activity. A specific gene expressed in an inducible manner exhibits several advantages for the applications of gene therapy and functional study, include reducing toxic effect, allowing temporal control and easy maintaining expression level. Among developed inducible expression systems tetracycline (Tet)-induced expression system is widely used in the expression of exogenous genes in different hosts, including embryonic stem cells (Kasuda *et al.*, 2008), mammalian cell culture (Howe *et al.*, 1995), transgenic mice (Ewald *et al.*, 1996), insect (Thomas *et al.*, 2000), and plant (Bohner *et al.*, 1999). The integration of issue-specific promoters allows Tet-inducible expression system to spatially control the expression of exogenous gene products in specific type of cells or tissues (Gunther *et al.*, 2002; Gallagher *et al.*, 2003; Michalon *et al.*, 2005).

The basic design for conventional Tet-inducible expression system is to separate the whole system into two distinct plasmids: a regulator unit for the constitutive expression of transactivator and a responsive unit under the control of tetracycline-responsive minimal promoter (tetO) for the transgene expression. The tetracycline-controlled transcriptional activator (tTA) and reverse tetracycline-controlled transcriptional activator (rtTA) are transactivators for the Tet-off and Tet-on

(1) Contribution No. 2567 from Livestock Research Institute, Council of Agriculture, Executive Yuan.
(2) Hsinchu Branch, COA-LRI, Sihoo, Miaoli, 36843, Taiwan, R.O.C.
(3) Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, 30068, Taiwan, R.O.C.
(4) Corresponding author, E-mail: jschao@mail.tlri.gov.tw.

inducible systems, respectively. In a Tet-on inducible system, the rtTA activated by doxycycline (Dox) and binds to TetO of expression plasmid to induce the expression of wanted gene. Typically, the generation of transgenic animals with transgene under the control of Tet-on system requires the cross of two transgenic strains. One transgenic strain carries the responsive unit of interested gene, which is under the control of tetO; while the other carries the transactivator unit (Ryding *et al.*, 2001). The whole process for the generation of a successful progeny containing a complete Tet-on inducible system is tedious, costly and time consuming.

High basal leakage and low induction rate are other disadvantages for the conventional Tet-mediated transgene induction system. To overcome these disadvantages the transactivator TetR is modified by fusing with the Krüppel-associated box (KRAB) domain, by which tetO can be completely blocked by TetR/KRAB fusion protein without induction. In the presence of tetracycline, TetR/KRAB fusion protein is inactivated, and allows transcription of transgene to occur (Forster *et al.*, 1999; Szulc *et al.*, 2006; Bulat and Widmann, 2008). The expression of transgenes is improved by simultaneously controlling the expression of transgene and rtTA via an internal ribosomal entry site (IRES) in an auto-regulatory manner (Hofmann *et al.*, 1996; Kuhnel *et al.*, 2004; Markusic *et al.*, 2005). It was also shown to modulate the gene expression of receptor for advanced glycation of end products (RAGE) in a two-plasmid Tet-on system by altering the ratio of regulatory and responsive plasmids (Shaikh and Nicholson, 2006).

Tet-inducible single vector systems containing both the transactivator rtTA and the tetracycline responsive element were proposed and constructed (Bäckman *et al.*, 2004; Zabala *et al.*, 2004; Bulat and Widmann, 2008). A single vector containing both regulatory and responsive elements, which are separated by a 5 kb intron from human p53 gene to reduce the cross interference, was proposed and constructed by Bäckman *et al.* (2004). However, the proposed inducible single vector system exhibits a great difficulty in transgene insertion and significantly low efficiency in the generation of transgenic animals. A tissue-specific Tet-inducible vector for transgene expression in β cell was also developed (Bulat and Widmann, 2008). However, an initial expression of both the regulator and transgene with low level is required for the efficient expression of the transgene, making it unsuitable for the study of gene product with high cytotoxic (Bulat and Widmann, 2008).

In this study, a single vector containing a complete Tet inducible expression system was constructed to avoid the disadvantages of the conventional two-vector based Tet-inducible expression system. The developed Tet-inducible vector was functional and exhibited a tight control in the transgene expression. Further study showed that the developed single vector-based inducible system could be easily adapted to pancreatic beta cells, with a high expression efficiency.

# Materials and Methods

## I. Reagents and plasmids

pTRE2hyg plasmid which contained the minimal RNA polymerase II promoter with multiple tetO, and pTet-tTS plasmid which contained a Tet-controlled transcriptional silencer tTS, and pMAMneo plasmid from Clontech Laboratories were employed for this study. The pUHrT62-1, a plasmid with rtTA2s-M2 cDNA, kindly provided by Dr. Hermann Bujard (Heidelberg University, Heidelberg, Germany) (Urlinger *et al.*, 2000). The RIP-vMos, a plasmid that contained a 405-bp fragment of RIP, donated from Dr. Bernard Thorens (Lausanne University, Lausanne, Switzerland). The PIP-TOPO, a plasmid that contained a 1500-bp fragment of PIP, donated from Dr. Marlon R. Schneider (Ludwig-Maximilians-University of Munich, Munich, Germany). Restriction enzymes purchased from New England Biolab. All other reagents were reagent grade.

## II. Construction of Plasmids

The ptTS/oRRT$_{neo}$/GFP and ptTS/oPRT$_{neo}$/GFP vector constructed for the specific expression of the transgene in pancreatic β-cells. Briefly, the RIP DNA fragment (405 bp in length) amplified by PCR using RIP-vMos plasmid as a template with the primer set BGL-PME-5-RIP405 (5'-agatctgtttaaacGGACACAGCTATCAG-3') and HIND-PME-3-RIP405 (5'-aagcttgtttaaacTAGGGCTGGGGGTTAC-3'). The MMTV promoter of pMRT$_{neo}$(-TRE2) (Chao *et al.*, 2012) between *Bgl*II and *Hin*dIII sites was then replaced with RIP to generate pRRT$_{neo}$(-TRE2). The ptTS/oRRT$_{neo}$/GFP constructed by inserting a 1.2 kb PmeI-NotI fragment into the *NruI-NotI* sites of ptTS/oMRT$_{neo}$/GFP (Fig. 1). A about 1,500 bp fragment of the 5' flanking region of the porcine insulin gene was amplified by PCR using PIP-TOPO plasmid as a

template with the primer set PIP-5'-*NruI* (5'-tcgcgaGAGTTCAGCTGAGCTGGCTC-3') and PIP-3'-Hind (5'-aagcttTG GGGGACGGGCGGCGTT-3'). The MMTV promoter of pMRT$_{neo}$(-TRE2) (Chao *et al.*, 2012) between *Nru*I and *Hin*dIII sites was then replaced with PIP to generate pPRT$_{neo}$(-TRE2). The ptTS/oPRT$_{neo}$/GFP constructed by inserting a 2.3 kb *Nru*I-*Not*I fragment into the *Nru*I-*Not*I sites of ptTS/oMRT$_{neo}$/GFP (Fig. 1).

**III. Cell Culture and Plasmid Transfection**

Duck kidney cells (DK) were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) contained 10% fetal bovine serum (FBS, Biological Industries, Israel), 100 units/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen, USA) in a 37ºC humidified incubator containing 95% air and 5% $CO_2$. Hamster pancreatic beta-cell (HIT-T15) was grown in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS) in a 37ºC humidified culturing chamber containing 95% air and 5% $CO_2$.

Plasmid transfection performed by using TurboFect (Thermo Fisher Scientific, USA) transfection kit according to the manufacturer's manual. Tet-on-inducible GFP expression in transfected cells monitored under fluorescent microscope and by Western blot analysis.

**IV. Western Blotting**

Cells were harvested and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin). Cell lysate (50 μg) was separated on a 10% SDS-PAGE and transferred to a PVDF membrane by electroblotting. The protein bands on membrane probed by primary antibodies specifically against actin and GFP (Santa Cruz Biotechnology, USA). The signals detected by an enhanced chemiluminescence system.

# Results and Discussion

A Tet-on inducible single vector (ptTS/oRRT$_{neo}$/GFP and ptTS/oPRT$_{neo}$/GFP) under the control of rat and porcine insulin promoter respectively, for the specific expression of a transgene in pancreatic β-cells, was constructed (Fig. 1). The Tet-on single DNA vector inducible systems contain a transactivator rtTA2s-M2 gene under the control of RIP or PIP promoter, a Tet-controlled transcriptional silencer tTS under the control of a CMV promoter, a GFP reporter gene under the control of tetO, which flanked with a TetCMVmin promoter, and a neomycin resistant gene.

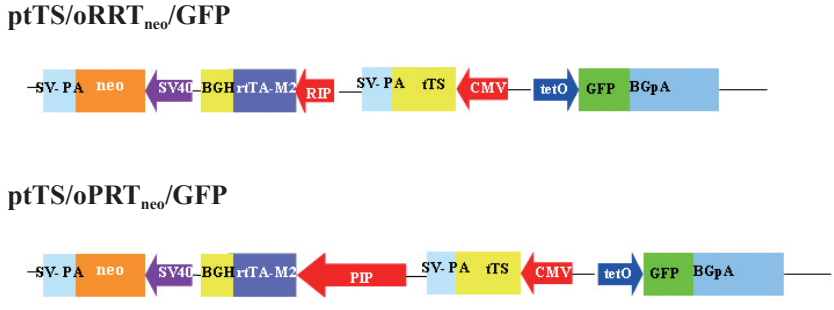


Fig. 1. Structure of RIP-based (ptTS/oRRT$_{neo}$/GFP) and PIP-based (ptTS/oPRT$_{neo}$/GFP) Tet-on inducible systems. The Tet-on single vector inducible systems contain a transactivator rtTA2s-M2 gene under the control of RIP or PIP promoter, a Tet-controlled transcriptional silencer tTS under the control of a CMV promoter, a GFP reporter gene under the control of tetO, which is flanked with a TetCMVmin promoter, and a neomycin resistant gene.

The basal expression of GFP was unobserved in HIT-T15, a rat pancreatic β-cell line, when without induction (Fig. 2). However, when treated with Dox at a concentration of 10 ng/mL or above, GFP expressed dose-dependently. The high basal expression of transgene in an inducible system may cause severe problems in model cells and animals, such as high background responses, low sensitivity of detection and cytotoxicity. Hence, it is important for an inducible transgene expression system to maintain the basal expression of transgenes as low as possible. Bulat and Widmann (2008) indicated their system that it requires a slight initial expression leakage of both the regulator and the sequence of interest. This might make it incompatible with sequences of interest that encode highly toxic molecules. Interestingly, our developed Tet-

inducible vectors display a negligible basal expression and high inducibility to Tet treatment.

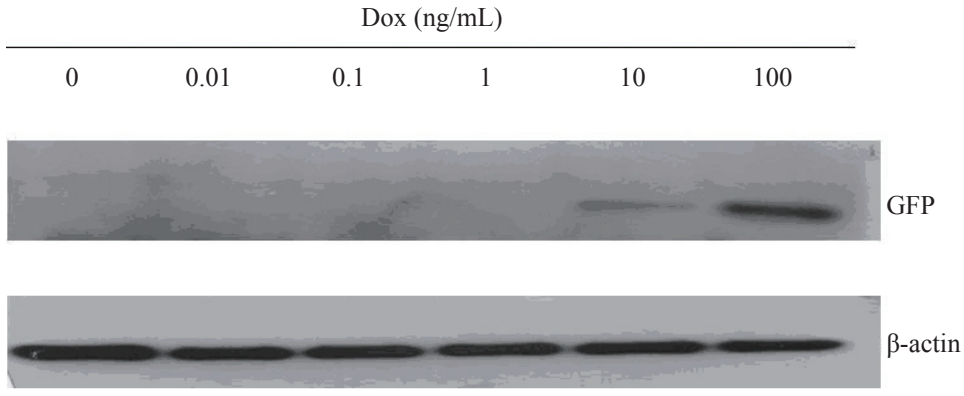


Fig. 2. Dox dose-dependent expression of GFP in ptTS/oRRT$_{neo}$/GFP-transfected HIT-T15 cells. The plasmid ptTS/oRRT$_{neo}$/GFP-transfected HIT-T15 cells ($1 \times 10^6$ cells) treated with various concentrations of Dox (0.01, 0.1, 1, 10 and 100 ng/mL) at 37°C for 24 h before cell lysate preparation. Western blot analysis performed by using the antibody specifically against GFP. β-Actin was used as an internal control.

Insulin gene promoter is also responsive to glucose in the regulation of transgene expression (Bäckman *et al.*, 2004). The main regulator of β cell function and insulin gene expression is glucose (Nielsen *et al.*, 1985; Evans-Molina *et al.*, 2007; Wang *et al.*, 2016). As shown in Fig. 3A, the expression of GFP in ptTS/oRRT$_{neo}$/GFP-tranfected HIT-T15 cells obviously increased in the high-glucose medium and in the presence of 10 ng/mL of Dox or above. In contrast, as shown in Fig. 3B, the expression of GFP in ptTS/oRRT$_{neo}$/GFP-tranfected HIT-T15 cells severely attenuated in the absence of glucose, even in the presence of 100 ng/mL of Dox. This result demonstrated that the developed insulin-gene-promoter-based Tet-on inducible DNA vector enabled the tight glucose-dependent expression of transgene.

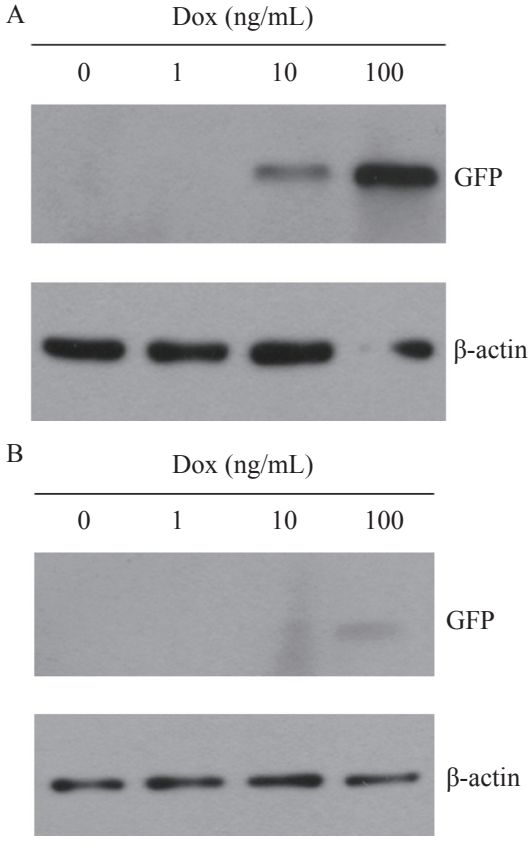


Fig. 3. The glucose-dependent tissue-specific expression of GFP in pancreatic β-cells by the simplified Tet-on inducible system. The ptTS/oRRT$_{neo}$/GFP -transfected HIT-T15 cells grew in culture medium with (A) or without (B) 4.5 g/L glucose. After transfection cells were treated with various concentrations of Dox (0, 1, 10 and 100 ng/mL) at 37°C for 24 h. The GFP expression in cells determined by Western blot analysis. β-Actin was used as an internal control.

To determine if β cell-specific expression of GFP could be obtained with our system, we also transfected our Tet-inducible expression system into a different cell type DK cells, a duck kidney cell line. The results shown that ptTS/oPRT$_{neo}$/GFP allowed strong Dox-dependent GFP expression in HIT-T15 cells. In contrast, it could not lead to the expression of GFP in DK cells, in the presence of Dox (Fig. 4).

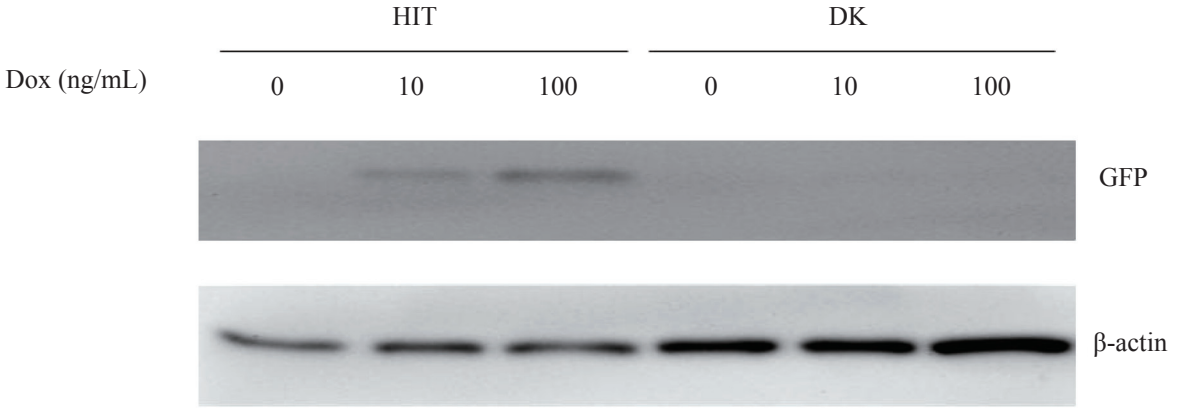


Fig. 4. Pancreatic β-cell-specific expression of GFP by the simplified Tet-on inducible vector ptTS/oPRT$_{neo}$/GFP. The ptTS/oPRT$_{neo}$/GFP-transfected HIT-T15 or DK cells treated with various concentrations of Dox (0, 10 and 100 ng/mL) at 37°C for 24 h in the culture medium containing 4.5 g/L glucose. The GFP expression in cells determined by Western blot analysis. β-Actin was used as an internal control.

In this study, a simplified Tet-inducible transgene expression system generated by integrating both regulatory and responsive elements in one vector. The developed single Tet-inducible expression vectors demonstrated to be feasible for the expression of transgene in β-cell hosts. Moreover, the presence of single cutting sites, MluI and SalI, in the developed Tet-inducible system in a single vector allows the size of linearized vector to shortened and, hence, increase the efficiency of transgenic animal generation. The developed single-vector based Tet-inducible expression system exhibits several advantages, such as simple, time-saving, easy-to-construction, versatility and high inducibility.

In the future, we will conduct *in vivo* or *ex vivo* pancreatic models to expressing transgene under the control of insulin gene promoter of our developed Tet-inducible system.

# Conclusion

This result revealed that the developed RIP- and PIP-based Tet-on inducible gene expression vector is capable of directing the expression of transgene in β-cell-type- and condition-specific manner. In addition, the developed Tet-inducible vector is also capable of carrying out the other cell-type specific expression of transgene by replacing the RIP or PIP promoter with a tissue specific promoter, such as leptin gene promoter and casein gene promoter.

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# 建立以胰島素基因啟動子啟動可誘導式基因表現之 DNA 載體系統 (1)

趙俊炫 (2)(4)　袁俊傑 (3)　賈玉祥 (2)

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## 摘　要

四環黴素可誘導系統 (tetracycline inducible system) 是目前廣泛使用於調節基因表現的系統之一，當添加四環黴素時即可誘發基因表現。本試驗構築一簡便 DNA 載體系統，具有可誘發性基因表現及 β- 細胞特異性表現，主要 DNA 原件含有可受大鼠及豬胰島素基因啟動子調控之四環黴素調節性 DNA 片段，以及可表現外源基因如綠螢光蛋白的四環黴素反應性 DNA 片段。將此 DNA 載體系統轉殖入大鼠胰臟 β- 細胞株後，添加四環黴素確實可有效誘發外源基因綠螢光蛋白的表現，也受培養液葡萄糖濃度所調控其表現，但此外源基因綠螢光蛋白卻不能被誘發表現於鴨腎臟細胞株。這些結果證明我們已經建構可應用於 β- 細胞特異性表現之可調節式基因專一表現系統。

關鍵詞：四環黴素可誘導系統、β- 細胞、基因調控。

(1) 行政院農業委員會畜產試驗所研究報告第 2567 號。
(2) 行政院農業委員會畜產試驗所新竹分所。
(3) 國立交通大學生物科技系。
(4) 通訊作者，E-mail：jschao@mail.tlri.gov.tw。